

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
14 December 2000 (14.12.2000)

PCT

(10) International Publication Number  
**WO 00/75353 A1**

(51) International Patent Classification<sup>7</sup>: C12N 15/86,  
5/10, 9/22, 7/01

[CN/US]; Apartment P4, 155 Washington Lane, Jenkin-  
town, PA 19046 (US). WILSON, James, M. [US/US];  
1350 N. Avignon Drive, Gladwyne, PA 19035 (US).

(21) International Application Number: PCT/US00/00415

(22) International Filing Date: 7 January 2000 (07.01.2000)

(74) Agents: KODROFF, Cathy, A. et al.; Howson and How-  
son, Spring House Corporate Center, P.O. Box 457, Spring  
House, PA 19477 (US).

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/137,105 2 June 1999 (02.06.1999) US

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ,  
BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK,  
DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL,  
IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,  
LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT,  
RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA,  
UG, US, UZ, VN, YU, ZA, ZW.

(71) Applicant (*for all designated States except US*):  
TRUSTEES OF THE UNIVERSITY OF PENNSYL-  
VANIA [US/US]; Center for Technology Transfer, Suite  
300, 3700 Market Street, Philadelphia, PA 19104-3147  
(US).

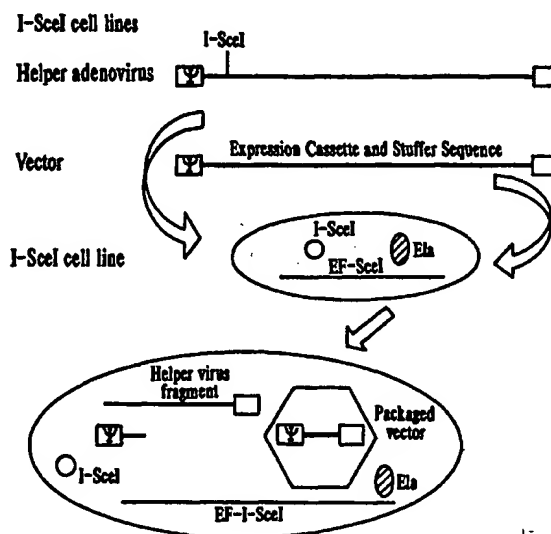
(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent  
(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent  
(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU,  
MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM,  
GA, GN, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): XIAO, Weidong

[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS USEFUL FOR PRODUCTION OF RECOMBINANT VIRUSES WHICH RE-  
QUIRE HELPER VIRUSES



(57) Abstract: A method of producing recombinant viruses which require helper viruses for packaging is provided. The method uses a recombinant helper virus which has been designed to contain at least one rare-cutting restriction site (e.g., for I-SceI), a viral vector which is helper-dependent for packaging, and a recombinant host cell which is capable of expressing a rare-cutting restriction enzyme (e.g., I-SceI). The method involves transfecting or infecting the host cell with the helper virus and viral vector and incubating the cell under conditions which permit encapsidation of the viral vector in an adenovirus capsid. Thereafter, the I-SceI expressed by the host cell digests the helper virus, permitting ready separation of the digested fragments of the helper virus from the packaged recombinant virus.

WO 00/75353 A1

WO 00/75353 A1



**Published:**

— *With international search report.*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

## COMPOSITIONS AND METHODS USEFUL FOR PRODUCTION OF RECOMBINANT VIRUSES WHICH REQUIRE HELPER VIRUSES

### Field of the Invention

5           The present invention relates generally to compositions and methods useful in producing recombinant viruses, and particularly, those methods which require the use of a helper virus. More particularly, the invention provides compositions and methods useful in obtaining a recombinant viral particle in a form which is readily separated from cultures in which helper virus was used in the production.

### 10   Background of the Invention

          The usefulness of traditional adenovirus vectors is often limited because of induction of strong cell-mediated immune response [Yang et al., *Gene Ther.*, 3:137-144 (1996)]. Previous studies with E1 deleted vector demonstrated that viral gene expression contributes to this problem [Yang et al, *J. Virol*, 70:7209-7212 (1996);  
15   Yang et al, *Immunity*, 1:433-442 (1994)]. Newly developed helper dependent adenovirus vectors, constructed through complete removal of all adenovirus coding regions [Mitani, *Proc. Natl. Acad. Sci. USA*, 92:3854-3858 (1995)], have become very promising gene therapy vectors since prolongation of transgene expression and increased capacity have been tested in a number of animal experiments [Fisher et al,  
20   *Virology*, 217:11-22 (1996); Morsy et al, *Proc. Natl. Acad. Sci. USA*, 95:7866-7871 (1998); Schiedner et al, *Nat. Genet.*, 18:180-183 (1998); Whittle, *Trends Genet.*, 14:136-137 (1998)]. However, such vectors make poor pharmaceutical quality vectors because helper virus contamination make them difficult to produce. Uncontrolled helper virus replication competes with the vector replication and  
25   packaging, which also decrease the yield. The commonly used cre-loxP system has been the primary system available to generate helper dependent adenovirus vectors with diminished helper virus [Parks et al, *Proc. Natl. Acad. Sci USA*, 93:13565-13570 (1996); Parks, *J. Virol.*, 71:3293-3298 (1997)].

          What are needed are methods of producing recombinant viruses which are  
30   substantially free of contamination with helper virus.

### Summary of the Invention

The present invention provides compositions and methods which permit production of recombinant viral vectors which are readily purified from helper viruses. The method uses a recombinant helper virus which has been designed to contain at least one site for a rare-cutting restriction enzyme, a viral vector which is helper-dependent for packaging into a virus, and a recombinant host cell which expresses the rare-cutting restriction enzyme. In a preferred embodiment, the rare-cutting enzyme is I-SceI. The method involves transfecting or infecting the host cell with the helper virus and viral vector and incubating the cell under conditions which permit packaging of the viral vector. Thereafter, the rare-cutting restriction enzyme expressed by the host cell digests the helper virus, permitting ready separation of the digested fragments of the helper virus from the packaged recombinant virus. In a preferred embodiment, the restriction enzyme is I-SceI and the viral vector is an adenovirus.

Thus, in one aspect, the present invention provides a method for producing helper-dependent virus which involves the following steps. A host cell capable of expressing a rare-cutting restriction enzyme (e.g., I-SceI) is provided. The host cell is transfected with a recombinant viral vector comprising a minigene containing a transgene encoding a selected protein and regulatory sequences which control expression of said protein. Subsequently, the host cell is infected with a recombinant helper virus engineered to contain at least one, and preferably multiple restriction sites for the rare-cutting restriction enzyme, e.g., I-SceI. The host cell with the recombinant viral vector and helper virus is then cultured under conditions which permit packaging of the recombinant viral vector in a viral particle, wherein the helper virus and the host cell provide to the recombinant adenovirus vector the necessary viral genes for viral packaging.

In another aspect, the present invention provides a recombinant virus produced by the method of the invention, which is substantially purified from helper virus.

In yet another aspect, the invention provides a stable mammalian cell line which expresses a rare-cutting restriction enzyme, e.g., I-SceI.

In still another aspect, the invention provides a recombinant helper virus useful in helper-dependent production of a recombinant virus vector, wherein said helper virus is engineered to contain a site for a rare-cutting restriction enzyme downstream of the packaging signal.

5 In still a further aspect, the invention provides a method for the helper-dependent production of a recombinant virus which involves transfecting a host cell with a recombinant viral vector comprising a minigene containing a transgene encoding a selected protein and regulatory sequences which control expression of said protein. The host cell is further infected with a recombinant helper virus engineered  
10 to contain a site for a rare-cutting enzyme, e.g., I-SceI, and the corresponding restriction enzyme, e.g., I-SceI, is delivered to the host cell. The host cell is then cultured under conditions which permit packaging of the recombinant virus vector in a viral particle. Suitably, the helper virus and the host cell provide to the recombinant viral vector the necessary viral genes for viral packaging and the  
15 restriction enzyme cleaves the recombinant helper virus following generation of the recombinant viral vector.

In yet a further aspect, the invention provides a cell lysate comprising a recombinant virus which is substantially free of helper virus.

Other aspects and advantages of the invention will be readily apparent from  
20 the following detailed description of the invention.

#### Brief Description of the Drawings

Fig. 1A is a schematic illustration of the generation of recombinant adenoviruses using I-SceI expressed from the human 293 cell line.

Fig. 1B is a schematic illustration of the generation of recombinant  
25 adenoviruses using recombinant AAV vectors to deliver the functional I-SceI enzymes

Fig. 2 is a detailed restriction map for a recombinant adenovirus helper virus of the invention, Ad-3I. There are three I-SceI sites, one located before the CMV promoter, one located between the CMV promoter and the  $\alpha$ 1-antitrypsin gene and

one located after the  $\alpha 1$ -antitrypsin gene, respectively. The CMV- $\alpha 1$ -antitrypsin cassette was located right after the 3' end of adenovirus genome.

Fig. 3 is a line graph providing a time course of gene expression from Ad-3I in the presence of I-SceI enzyme in human 293 cells. Ad-3I (MOI 10) infected either 293 cells, 293-I-SceI cell line or 293 cells along with rAAV-I-SceI at MOI 10. The amount of human  $\alpha 1$ -antitrypsin in media was measured by ELISA at the various times indicated in the graph.

Fig. 4 is a bar chart illustrating that 293 I-SceI cell lines enhanced helper dependent adenovirus production.

Fig. 5 is a bar graph illustrating the performance of an I-SceI cell line of the invention, as determined by helper virus at 48 hours post-infection shown as the  $\alpha 1$ -antitrypsin secreted into the media. The digested helper virus is not able to express  $\alpha 1$ -antitrypsin. The same amount of Ad- $\Delta$ -LacZ vector and Ad-3I mix were used to infect the same amount of 293, 293-I-SceI(a), 293-I-SceI(b) cells. The viruses were harvested 48 hours post-infection. The lacZ-forming unit (LFU) was determined by X-Gal staining.

#### Detailed Description of the Invention

The present invention provides compositions and methods which permit production of recombinant viral vectors which are readily separated from helper viruses. Thus, the invention is particularly suited for production of viral vectors which are dependent upon helper viruses for packaging and/or encapsidation into a recombinant virus. The invention uses a recombinant helper virus which has been designed to contain at least one, and preferably, multiple sites for a selected rare-cutting restriction enzyme and a recombinant host cell which is capable of expressing the rare-cutting restriction enzyme. As used herein, a helper virus is any virus which, in conjunction with the selected host cell, provides the necessary viral products to permit packaging and/or encapsidation of the selected viral vector into an infectious recombinant virus.

The host cell may be a cell line stably or transiently expressing the rare-cutting restriction enzyme. The method involves delivering the helper virus and viral

vector to a host cell which expresses the restriction enzyme (or is modified to express the enzyme) and culturing the cell under conditions which permit encapsidation of the viral vector. Thereafter, the restriction enzyme expressed by the host cell digests the helper virus, permitting ready separation of the digested fragments of the helper virus  
5 from the packaged recombinant virus.

As defined herein, a rare-cutting restriction enzyme is a restriction enzyme which is not naturally present in the selected host cell and/or selected viral vector genome or which recognizes a site which is present sufficiently infrequently in the genomes of the species of the host or virus, that the site (and/or enzyme) is unlikely to  
10 occur naturally in the host cell or viral vector. In preferred embodiment, a suitable rare-cutting enzyme for use in the present invention is the restriction enzyme I-SceI.

The I-SceI enzyme is an endonuclease encoded by the group I intron of *S. cerevisiae* mitochondria [L. Colleaux et al, *Proc. Natl. Acad. Sci. USA*, 85:6022-6026 (1988), which has high specificity for an 18 bp nonpalindromic nucleotide sequence  
15 [I-SceI site: SEQ ID NO:1: 5'-tagggataa/cagggtaat]. In a human genome with about  $3 \times 10^9$  nucleotides, a common restriction endonuclease generally recognizes a short stretch of nucleotides of 4 to 8 base pairs; thus, there would be about one million such sites in one human genome. In contrast, the I-SceI site occurs randomly only once in every 20 human genomes. The rarity of I-SceI sites has been partially confirmed by  
20 the fact that there are no I-SceI sites in the genomes of many organisms, including viruses, bacteria and yeast. Based on the information provided herein, one of skill in the art can readily substitute other suitable rare-cutting restriction enzymes for I-SceI for use in the invention.

Such rare-cutting restriction enzymes may be selected, for example, from  
25 among various restriction enzymes which are native to non-mammalian animals, plants, yeast, fungi, and/or insects, which restriction enzymes are not native to mammalian species. Examples of rare-cutting restriction enzymes, in addition to I-SceI include, PspI, I-CeuI, and the like. Other suitable rare-cutting restriction enzymes may be selected from among those which recognized sites which are at least  
30 about 12 to about 40 nucleotides in length, preferably about 14 to about 20 nucleotides in length, and most preferably, at least about 18 nucleotides in length.

Such restriction enzymes are available from a variety of commercial sources, including, e.g., New England BioLabs, Promega, and Boehringer Mannheim. Alternatively, these enzymes or their coding sequences may be produced synthetically or using recombinant technology.

5           For example, the I-SceI enzyme may be purchased from commercial sources (e.g., Boehringer Mannheim, Germany). Alternatively, the sequence of the enzyme may be produced by conventional chemical synthesis. [See, e.g., G. Barony and R.B. Merrifield, *The Peptides: Analysis, Synthesis & Biology*, Academic Press, pp. 3-285 (1980)]. Preferably, the native coding sequence for this enzyme (or another selected  
10   rare-cutting restriction enzyme) is altered to optimize expression in mammalian cells, which are the preferred host cells. Techniques for optimizing expression, e.g., by altering preference codons, are well known to those of skill in the art. Similarly, the sequences for the I-SceI site and, other selected rare-cutting restriction enzyme sites, may be produced synthetically, recombinantly, or obtained using other suitable  
15   techniques. See, e.g., Barony and Merrifield, cited above; Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.

## **I. Host Cells**

The invention provides host cells which are useful in the production of helper-  
20   dependent viruses. In one embodiment, the invention provides cell lines which stably express a rare-cutting restriction enzyme. In another embodiment, the sequences encoding the rare-cutting restriction enzyme are delivered in *trans* to a selected host cell via a suitable vector or other nucleic acid molecule. For convenience throughout this specification, reference will be made to the I-SceI enzyme. However, it will be  
25   readily understood that another rare-cutting restriction enzyme and/or its restriction enzyme site, as defined herein, may be substituted.

### **A. Stable Cell Line Expressing Rare-Cutting Restriction Enzyme Functions**

A cell line of the invention may be constructed by providing the  
30   selected host cell line with a nucleic acid molecule encoding a rare-cutting restriction



enzyme or a functional fragment thereof operably linked to regulatory sequences which control expression thereof using conventional techniques. As used herein, "operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in *trans* or at a distance to control the gene of interest.

Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A great number of expression control sequences, native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized.

Desirably, the nucleic acid molecule encoding the rare-cutting restriction enzyme, e.g., I-SceI, is further provided with a nuclear localization signal, which targets the I-SceI sequences to the nucleus. Suitable nuclear localization signals are known to those of skill in the art and are not a limitation of the present invention.

In one embodiment, the host cell stably expresses the rare-cutting restriction enzyme, e.g., I-SceI, under the control of a constitutive promoter. Examples of such promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al, *Cell*, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the  $\beta$ -actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1 $\alpha$  promoter [Invitrogen].

In another embodiment, the rare-cutting restriction enzyme, e.g., I-SceI, is stably expressed by the host cell under the control of an inducible promoter. Inducible promoters are regulated by exogenously supplied compounds, including, the zinc-inducible sheep metallothioneine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase

promoter system [WO 98/10088]; the ecdysone insect promoter [No et al, *Proc. Natl. Acad. Sci. USA*, **93**:3346-3351 (1996)], the tetracycline-repressible system [Gossen et al, *Proc. Natl. Acad. Sci. USA*, **89**:5547-5551 (1992)], the tetracycline-inducible system [Gossen et al, *Science*, **268**:1766-1769 (1995), see also Harvey et al, *Curr. Opin. Chem. Biol.*, **2**:512-518 (1998)], the RU486-inducible system [Wang et al, *Nat. Biotech.*, **15**:239-243 (1997) and Wang et al, *Gene Ther.*, **4**:432-441 (1997)] and the rapamycin-inducible system [Magari et al, *J. Clin. Invest.*, **100**:2865-2872 (1997)].

Most preferably, the cell line selected for transformation with the nucleic acid molecule encoding the rare-cutting restriction enzyme, e.g., I-SceI, and, optionally, expression control sequences therefor, is a mammalian cell line, including, without limitation, cells such as A549, WEHI, 3T3, 10T1/2, BHK, MDCK, COS1, COS7, BSC 1, BSC 40, BMT 10, VERO, WI38, HeLa, Saos, C2C12, L cells, HT1080, HepG2 and primary fibroblast, hepatocyte and myoblast cells derived from mammals including human, monkey, mouse, rat and hamster. Preferred cells include human cells, and most preferably, cells which express adenovirus E1 functions, e.g., 293 cells. However, other cell lines may be readily obtained from the American Type Culture Collection (ATCC) or a variety of commercial and academic sources.

#### B. *Delivery of Rare-cutting Restriction Enzyme*

In another embodiment, the method of the invention is performed by delivery of sequences encoding the rare-cutting restriction enzyme, e.g., I-SceI, or a functional fragment thereof to a selected host cell in *trans*. The nucleic acid molecule carrying the rare-cutting restriction enzyme (e.g., I-SceI) coding sequences and expression control sequences may be in any form which transfers these components to the host cell and permits expression of the restriction enzyme, preferably in the nucleus. In this embodiment, the selected host cell is preferably a human cell line, e.g., 293. However, other suitable host cells may be readily selected from among those known in the art. See discussion of host cells above.

The nucleic acid molecule carrying the sequences encoding the rare-cutting restriction enzyme (e.g., I-SceI), as well as the sequences which regulate expression thereof, are provided to the host cell by any suitable method, including transfection, electroporation, liposome delivery, membrane fusion techniques, high

velocity DNA-coated pellets, viral infection and protoplast fusion. See, for instance, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, New York. Most suitably, these sequences are contained within a vector. A "vector" includes, without limitation, any genetic  
5 element, such as a plasmid, phage, transposon, cosmid, chromosome, virus, virion, etc..

In one desirable embodiment, the restriction enzyme (e.g., I-SceI) coding sequences are delivered via a viral vector, and most preferably, via a recombinant, infectious virus. Selection of the enzyme delivery virus is not a  
10 limitation on the present invention. Suitable recombinant enzyme (e.g., I-SceI) delivery viruses may be readily engineered utilizing such viruses as adeno-associated viruses (AAV), retroviruses, adenoviruses, hybrid adeno-AAV viruses, lentiviruses, baculovirus, herpes virus, and pox viruses, among others.

In one currently preferred embodiment, the enzyme delivery virus is a  
15 recombinant AAV (rAAV) containing the enzyme coding sequences operably linked to suitable expression control sequences, which direct expression of the enzyme (e.g., I-SceI) in the host cell and target the nucleus. This I-SceI-rAAV and other delivery virus constructs of the invention are prepared using the rare-cutting restriction enzyme sequences, obtained as described herein, and using known methods. For  
20 example, methods for producing rAAV vectors have been described. [See, W. Xiao et al, *J. Virol.*, 72:10222-10226 (1998); US Patent No. 5,658,776; US Patent No. 5,622,856, among others].

Generally, an I-SceI-rAAV of the invention employs the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences [see, e.g, B.J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp. 155-168 (1990)] flanking the  
25 sequences encoding I-SceI and directing expression thereof. The ITR sequences are about 145 bp in length. Preferably, substantially the entire sequences encoding the ITRs are used in the molecule, although some degree of minor modification of these sequences is permissible. The ability to modify these ITR sequences is within the  
30 skill of the art. [See, e.g., texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual.", 2d edit., Cold Spring Harbor Laboratory, New York (1989);

Carter et al, cited above; and K. Fisher *et al.*, J. Virol., 70:520-532 (1996)]. An example of such a molecule employed in the present invention is a "cis-acting" plasmid containing the transgene, in which the selected transgene sequence and associated regulatory elements are flanked by the 5' and 3' AAV ITR sequences.

5           The AAV ITR sequences may be obtained from any known AAV, including presently identified human AAV types. Similarly, AAVs known to infect other animals may also provide these ITRs employed in the molecules or constructs of this invention. For example, the ITRs may be provided by AAV type 1, AAV type 2, AAV type 3, AAV type 4, AAV type 5, parvovirus type H1, MVM, LuIII, or by  
10 any other parvovirus or AAV serotype. A variety of AAV strains are available from the American Type Culture Collection or are available by request from a variety of commercial and institutional sources. In the following exemplary embodiments an AAV-2 is used for convenience. However, the selection of the species and serotype of AAV that provides these sequences is within the skill of the artisan according to  
15 the teachings of this application and does not limit the following invention.

          In addition, the enzyme delivery virus contains the restriction enzyme nucleic acid sequences, a nuclear localization signal, and conventional regulatory elements necessary to drive expression of the enzyme in a host cell transfected (infected) with this enzyme delivery virus. Such expression control elements include  
20 promoters, including both constitutive and inducible promoters, as are described above, poly A sequences, and the like. Other heterologous nucleic acid sequences optionally present in this enzyme delivery virus include sequences providing signals required for efficient polyadenylation of the RNA transcript, and introns with functional splice donor and acceptor sites. A common poly-A sequence which is  
25 employed in the enzyme delivery viruses useful in this invention is that derived from the papovavirus SV-40. In the I-SceI-rAAV delivery virus, the poly-A sequence generally is inserted following the transgene sequences and before the 3' AAV ITR sequence. An enzyme delivery virus useful in the present invention may also contain an intron, desirably located between the promoter/enhancer sequence and the  
30 transgene. One possible intron sequence is also derived from SV-40, and is referred

to as the SV-40 T intron sequence. Selection of these and other common vector elements are conventional and many such sequences are available [see, e.g., Sambrook et al, and references cited therein at, for example, pages 3.18-3.26 and 16.17-16.27]. Optionally, the enzyme delivery virus may contain a selectable marker or reporter sequences, such as sequences encoding hygromycin or purimycin, among others. See the discussion of reporter sequences below.

The engineering methods used to construct any embodiment of this invention are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, e.g., Sambrook et al, cited above; and International Patent Application NO. WO95/13598.

Suitably, the I-SceI-rAAV may be delivered to the selected host cells at a multiplicity of infection (MOI) of about 5 to about 200 rAAV genome particles, and preferably at an MOI of 10 to 100 rAAV genome particles. Suitable MOI for other selected enzyme delivery viruses may be in this range, or may be adjusted as desired by one of skill in the art. Alternatively, where the I-SceI is delivered by a vector which lacks the ability to infect host cells, the vector is delivered to the host cells in an amount of about 5 µg to about 100 µg DNA by any suitable means known to those of skill in the art.

The enzyme delivery vehicle (e.g., I-SceI-rAAV) may be provided to the host cells at any time prior to cell lysis, including prior to delivery of the vector (e.g., by infection or transfection), prior to delivery of the helper virus sequences (e.g., infection with the helper virus), or after delivery of either or both of these components to the host cell. For example, where the enzyme delivery vehicle constitutively expresses the restriction enzyme (e.g., I-SceI), it may be desirable to provide this enzyme delivery vehicle to the host cells following delivery of the viral vector and the helper virus. Alternatively, where the enzyme delivery vehicle inducibly expresses the restriction enzyme, the timing of delivery of the enzyme may not be critical. However, the selection of promoters, and the determination of timing of delivery of an enzyme delivery virus (or other nucleic acid molecule) to the host cell may be made by one of skill in the art in view of the information provided herein.

## II. Recombinant Vector

The compositions and methods of the present invention are particularly well suited for packaging of a recombinant viral vector which lacks sufficient viral genes to permit encapsidation in a capsid in the absence of a helper virus and host cell.

5 Together, the helper virus and host cell provide the gene functions necessary to encapsidate the recombinant virus. This present invention may be utilized for any viral vector which is dependent upon a helper virus for packaging. Such viral vectors may include, without limitation, recombinant adeno-associated vectors (rAAV), recombinant adenovirus vectors, hybrid adenovirus/AAV vectors, retroviruses, and  
10 lentiviruses. Selection of a suitable viral vector is not a limitation of the present invention.

In one embodiment, the method of the invention is particularly well suited for packaging of adenoviral vectors. In a preferred embodiment, the adenoviral vector contains only minimal adenovirus sequences. In one example, the recombinant  
15 adenovirus vector, referred to herein as pAdΔ is described below. Methods of producing these recombinant adenoviral vectors are known in the art. See, e.g., International Patent Publication No. WO96/13597.

### A. *The Adenovirus Sequences of pAdΔ*

The adenovirus nucleic acid sequences of the pAdΔ vector provide the  
20 minimum adenovirus sequences which enable a viral particle to be produced with the assistance of a helper virus and, optionally, a packaging cell line. These sequences assist in delivery of a recombinant transgene genome to a target cell by the resulting recombinant virus.

The DNA sequences of a number of adenovirus types are available  
25 from Genbank, including type Ad5 [Genbank Accession No. M73260]. The adenovirus sequences may be obtained from any known adenovirus serotype, such as serotypes 2, 3, 4, 7, 12 and 40, and further including any of the presently identified human types [see, e.g., Horwitz, cited above]. Similarly adenoviruses known to infect other animals may also be employed in the vector constructs of this invention.  
30 The selection of the adenovirus type is not anticipated to limit the following invention. A variety of adenovirus strains are available from the American Type

Culture Collection, Manassas, Virginia, or available by request from a variety of commercial and institutional sources. In the following exemplary embodiment, an adenovirus type 5 (Ad5) is used for convenience.

However, it is desirable to obtain a variety of pAdΔ vectors based on  
5 different human adenovirus serotypes. It is anticipated that a library of such plasmids and the resulting AdΔ viruses would be useful in a therapeutic regimen to evade cellular, and possibly humoral, immunity, and lengthen the duration of transgene expression, as well as improve the success of repeat therapeutic treatments. Additionally the use of various serotypes is anticipated to produce recombinant  
10 viruses with different tissue targeting specificities.

Specifically, the pAdΔ vector lacks nucleic acid sequences encoding all functional adenoviral genes. Such functional genes include E1, E2, E3, E4, the intermediate genes (IVa and IX) and late genes (L1, L2, L3, L4, L5). More specifically, the adenovirus sequences employed are the cis-acting 5' and 3' inverted  
15 terminal repeat (ITR) sequences of an adenovirus (which function as origins of replication) and the native 5' packaging/enhancer domain that contains sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter. These sequences are necessary for replication and virion encapsidation. See, e.g., P. Hearing et al, *J. Virol.*, 61(8):2555-2558 (1987); M. Grable and P.  
20 Hearing, *J. Virol.*, 64(5): 2047-2056 (1990); and M. Grable and P. Hearing, *J. Virol.*, 66(2):723-731 (1992).

The entire adenovirus 5' sequence containing the 5' ITR and packaging/enhancer region can be employed as the 5' adenovirus sequence in the pAdΔ vector. This left terminal (5') sequence of the Ad5 genome useful in this  
25 invention spans bp 1 to about 360 of the conventional adenovirus genome, also referred to as map units 0-1 of the viral genome. This sequence includes the 5' ITR and the packaging/enhancer domain. Preferably, this adenovirus 5' region is employed in the vector in its native, unmodified form. However, some modifications including deletions, substitutions and additions to this sequence which do not  
30 adversely effect its biological function may be acceptable. See, e.g., International Patent Publication No. WO 93/24641, published December 9, 1993. The ability to

modify these ITR sequences is within the ability of one of skill in the art. See, e.g., texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual.", 2d edit., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

The 3' adenovirus sequences of the vector include the right terminal  
5 (3') ITR sequence of the adenoviral genome spanning about bp 35,353 - end of the adenovirus genome, or map units ~98.4-100. This entire sequence is desirably employed as the 3' sequence of a pAdΔ vector. Preferably, the native adenovirus 3' region is employed in the shuttle vector in unmodified form. However, some modifications to this sequence which do not adversely effect its biological function  
10 may be acceptable.

An exemplary pAdΔ vector used in this invention, described below, lacks adenovirus sequences encoding functional adenoviral genes. The pAdΔ vector contains Ad5 5' and 3' cis-elements, as well as the transgene sequences described below. Suitably, these 5' and 3' elements may flank the transgene (e.g., 5' cis-  
15 elements, transgene, 3' cis-elements). Alternatively, these 5' ITRs and 3' ITRs may be oriented in a head-to-tail configuration, located upstream of the transgene. Such a vector may be constructed using conventional genetic engineering techniques, e.g., homologous recombination and the like. See, e.g., US Patent No. 6,001,557.

From the foregoing information, it is expected that one of skill in the  
20 art may employ other equivalent adenovirus sequences for use in the pAdΔ vectors of this invention. These sequences may include other adenovirus strains, or the above mentioned cis-acting sequences with minor modifications. Further, one of skill in the art will readily appreciate that the method of the invention is useful in overcoming difficulties in separating helper virus from production cultures for other viral vectors  
25 lacking gene functions required for packaging. Such other vectors include rAd vectors lacking one or more adenoviral genes selected from E1, E2, E4, the intermediate genes and the late genes, as well as non-adenoviral vectors. Other non-adenoviral vectors which require helper functions for production include, without limitation, parvoviruses, including adeno-associated viruses and retroviruses,  
30 including, lentiviruses. One of skill in the art can readily select other suitable systems for use of the present invention.



B. *The Transgene*

The transgene sequence of the recombinant vector and the virus resulting from the method of the invention is a nucleic acid sequence, heterologous to the virus sequence, which encodes a polypeptide, protein, or other product, of interest.

- 5 The transgene is operatively linked to regulatory components in a manner which permits transgene transcription.

The composition of the transgene sequence will depend upon the use to which the resulting virus will be put. For example, one type of transgene sequence includes a reporter sequence, which upon expression produces a detectable signal.

- 10 Such reporter sequences include without limitation, DNA sequences encoding  $\beta$ -lactamase,  $\beta$ -galactosidase (LacZ), alkaline phosphatase, thymidine kinase, green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), luciferase, membrane bound proteins including, for example, CD2, CD4, CD8, the influenza hemagglutinin protein, and others well known in the art, to which high affinity  
15 antibodies directed thereto exist or can be produced by conventional means, and fusion proteins comprising a membrane bound protein appropriately fused to an antigen tag domain from, among others, hemagglutinin or Myc.

- These sequences, when associated with regulatory elements which drive their expression, provide signals detectable by conventional means, including  
20 enzymatic, radiographic, colorimetric, fluorescence or other spectrographic assays, fluorescent activating cell sorting assays and immunological assays, including enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and immunohistochemistry. For example, where the marker sequence is the LacZ gene, the presence of helper virus is detected by assays for beta-galactosidase activity.  
25 Where the transgene is luciferase, the helper virus may be measured by light production in a luminometer.

- However, desirably, the transgene is a non-marker sequence encoding a product which is useful in biology and medicine, such as proteins, peptides, anti-sense nucleic acids (e.g., RNAs), enzymes, or catalytic RNAs. The transgene may be  
30 used to correct or ameliorate gene deficiencies, which may include deficiencies in which normal genes are expressed at less than normal levels or deficiencies in which

- the functional gene product is not expressed. A preferred type of transgene sequence encodes a therapeutic protein or polypeptide which is expressed in a host cell. The invention further includes using multiple transgenes, e.g., to correct or ameliorate a gene defect caused by a multi-subunit protein. In certain situations, a different
- 5 transgene may be used to encode each subunit of a protein, or to encode different peptides or proteins. This is desirable when the size of the DNA encoding the protein subunit is large, e.g., for an immunoglobulin, the platelet-derived growth factor, or a dystrophin protein. In order for the cell to produce the multi-subunit protein, a cell is infected with the recombinant virus containing each of the different subunits.
- 10 Alternatively, different subunits of a protein may be encoded by the same transgene. In this case, a single transgene includes the DNA encoding each of the subunits, with the DNA for each subunit separated by an internal ribozyme entry site (IRES). This is desirable when the size of the DNA encoding each of the subunits is small, e.g., the total size of the DNA encoding the subunits and the IRES is less than five kilobases.
- 15 However, the selected transgene may encode any product desirable for study. The selection of the transgene sequence is not a limitation of this invention.

Useful products encoded by the transgene include hormones and growth and differentiation factors including, without limitation, insulin, glucagon, growth hormone (GH), parathyroid hormone (PTH), growth hormone releasing factor

20 (GRF), follicle stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), vascular endothelial growth factor (VEGF), angiopoietins, angiostatin, granulocyte colony stimulating factor (GCSF), erythropoietin (EPO), connective tissue growth factor (CTGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), epidermal growth

25 factor (EGF), transforming growth factor  $\alpha$  (TGF $\alpha$ ), platelet-derived growth factor (PDGF), insulin growth factors I and II (IGF-I and IGF-II), any one of the transforming growth factor  $\beta$  superfamily, including TGF  $\beta$ , activins, inhibins, or any of the bone morphogenic proteins (BMP) BMPs 1-15, any one of the heregluin/neuregulin/ARIA/neu differentiation factor (NDF) family of growth factors,

30 nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins NT-3 and NT-4/5, ciliary neurotrophic factor (CNTF), glial cell line derived

neurotrophic factor (GDNF), neurturin, agrin, any one of the family of semaphorins/collapsins, netrin-1 and netrin-2, hepatocyte growth factor (HGF), ephrins, noggin, sonic hedgehog and tyrosine hydroxylase.

Other useful transgene products include proteins that regulate the immune system including, without limitation, cytokines and lymphokines such as thrombopoietin (TPO), interleukins (IL) IL-1 through IL-17, monocyte chemoattractant protein, leukemia inhibitory factor, granulocyte-macrophage colony stimulating factor, Fas ligand, tumor necrosis factors  $\alpha$  and  $\beta$ , interferons  $\alpha$ ,  $\beta$ , and  $\gamma$ , stem cell factor, flk-2/flt3 ligand. Gene products produced by the immune system are also useful in the invention. These include, without limitations, immunoglobulins IgG, IgM, IgA, IgD and IgE, chimeric immunoglobulins, humanized antibodies, single chain antibodies, T cell receptors, chimeric T cell receptors, single chain T cell receptors, class I and class II MHC molecules, as well as engineered immunoglobulins and MHC molecules. Useful gene products also include complement regulatory proteins such as complement regulatory proteins, membrane cofactor protein (MCP), decay accelerating factor (DAF), CR1, CF2 and CD59.

Still other useful gene products include any one of the receptors for the hormones, growth factors, cytokines, lymphokines, regulatory proteins and immune system proteins. The invention encompasses receptors for cholesterol regulation, including the low density lipoprotein (LDL) receptor, high density lipoprotein (HDL) receptor, the very low density lipoprotein (VLDL) receptor, and the scavenger receptor. The invention also encompasses gene products such as members of the steroid hormone receptor superfamily including glucocorticoid receptors and estrogen receptors, Vitamin D receptors and other nuclear receptors. In addition, useful gene products include transcription factors such as *jun*, *fos*, max, mad, serum response factor (SRF), AP-1, AP2, *myb*, MyoD and myogenin, ETS-box containing proteins, TFE3, E2F, ATF1, ATF2, ATF3, ATF4, ZF5, NFAT, CREB, HNF-4, C/EBP, SP1, CCAAT-box binding proteins, interferon regulation factor (IRF-1), Wilms tumor protein, ETS-binding protein, STAT, GATA-box binding proteins, e.g., GATA-3, and the forkhead family of winged helix proteins.

Other useful gene products include, carbamoyl synthetase I, ornithine transcarbamylase, arginosuccinate synthetase, arginosuccinate lyase, arginase, fumarylacetate hydrolase, phenylalanine hydroxylase, alpha-1 antitrypsin, glucose-6-phosphatase, porphobilinogen deaminase, factor VIII, factor IX, cystathione beta-synthase, branched chain ketoacid decarboxylase, albumin, isovaleryl-coA dehydrogenase, propionyl CoA carboxylase, methyl malonyl CoA mutase, glutaryl CoA dehydrogenase, insulin, beta-glucosidase, pyruvate carboxylate, hepatic phosphorylase, phosphorylase kinase, glycine decarboxylase, H-protein, T-protein, a cystic fibrosis transmembrane regulator (CFTR) sequence, and a dystrophin cDNA sequence.

Other useful gene products include, non-naturally occurring polypeptides, such as chimeric or hybrid polypeptides having a non-naturally occurring amino acid sequence containing insertions, deletions or amino acid substitutions. For example, single-chain engineered immunoglobulins could be useful in certain immunocompromised patients. Other types of non-naturally occurring gene sequences include antisense molecules and catalytic nucleic acids, such as ribozymes, which could be used to reduce overexpression of a gene. Other suitable transgenes may be readily selected by one of skill in the art. The selection of the transgene is not considered to be a limitation of this invention.

#### 20 C. *Regulatory Elements*

In addition to the major elements identified above for the viral vector, (e.g, the adenovirus sequences and the transgene), the vector also includes conventional control elements necessary to drive expression of the transgene in a cell transfected with the viral vector. Thus the vector contains a selected promoter which is linked to the transgene and located, with the transgene, between the viral sequences of the vector. Suitable promoters may be readily selected from among constitutive and inducible promoters, such as those discussed herein. Selection of these and other common vector elements are conventional and many such sequences are available [see, e.g., Sambrook et al, and references cited therein].

30 The combination of the transgene, promoter/enhancer, and the other regulatory vector elements is referred to as a "minigene" for ease of reference herein.

In a preferred embodiment, the minigene is flanked by the 5' and 3' cis-acting adenovirus sequences described above. Such a minigene may have a size in the range of several hundred base pairs up to about 30 kb due to the absence of adenovirus early and late gene sequences in the vector. Thus, this viral vector (e.g., pAdΔ) system permits a great deal of latitude in the selection of the various components of the minigene, particularly the selected transgene, with regard to size. Provided with the teachings of this invention, the design of such a minigene can be made by resort to conventional techniques.

D. *Delivery of Recombinant Vector to Host Cell*

In the performance of the method of the invention, the recombinant viral vector, e.g., pAdΔ, is delivered to the host cells using conventional techniques. Delivery can be by any suitable method, including transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion.

Currently, transfection is a particularly desirable method of delivering the viral vector (e.g., pAdΔ) to the host cell. One suitable method is described in Fisher et al, *Virol.*, **217**:11-22 (1996), which is incorporated by reference herein. Particularly, the pAdΔ is linearized by a suitable restriction enzyme and added in transfection cocktail. The transfection is then performed using the calcium-phosphate based techniques described in Cullen, in "Methods in Enzymology", ed. S.L. Berger and A.R. Kimmel, Vol. 152, pp. 684-704, Academic Press, San Diego (1987). Other suitable transfection techniques are known and may readily be utilized to deliver the recombinant vector to the host cell.

Generally, when delivering the recombinant vector (e.g., pAdΔ) by transfection, the vector is delivered in an amount from about 5 μg to about 100 μg DNA, and preferably about 10 to about 50 μg DNA to about  $1 \times 10^4$  cells to about  $1 \times 10^{13}$  cells, and preferably about  $10^5$  cells. However, the relative amounts of vector DNA to host cells may be adjusted, taking into consideration such factors as the selected vector, the delivery method and the host cells selected.

### III. Helper Virus

The present invention utilizes a recombinant helper virus for production of the viral vector, which helper virus has been engineered to contain heterologous restriction sites for the rare-cutting restriction enzyme, e.g., I-SceI. Normally, the production of a recombinant virus which utilizes helper virus containing a full complement of required genes results in recombinant virus contaminated by excess production of the helper virus. Thus, extensive purification of the recombinant virus from the contaminating helper virus is required. However, the present invention provides a way to facilitate purification and reduce contamination by enzymatic digestion of the helper virus by the rare-cutting restriction enzyme (e.g., I-SceI) expressed by the host cell. Thus, the helper viruses of the invention contain at least one, and preferably multiple, rare-cutting restriction enzyme sites, which are located within the genome of the helper virus such that the digested helper virus is composed of fragments which are small enough to be readily distinguishable from the recombinant virus and any nucleic acid used to deliver the restriction enzyme.

Generally, a helper virus is utilized in the production of a recombinant virus from a vector which contains insufficient viral genes to package (or encapsidate) the vector. Because of the limited amount of viral sequence present in the viral vector, a helper virus of this invention must, alone or in concert with a packaging cell line, provide the gene sequences necessary for a productive viral infection. A suitable helper virus may be readily selected by one of skill in the art, taking into consideration the viral vector to be packaged. For example, an AAV vector may be packaged in either a rAAV or a recombinant adenovirus (Ad) capsid, using a rAAV or Ad helper virus of a selected serotype, as appropriate. However, this and other vectors may be packaged using other selected helper viruses. Suitably, the helper viruses used in the present invention are capable of replication in the selected host cell. In one desirable embodiment, the helper viruses are replication-competent in the selected host cells and replication-incompetent in other cells, which do not provide the necessary gene functions to permit replication.

In one preferred embodiment, a pAdΔ vector is packaged into an adenoviral capsid using helper viruses which contain selected adenovirus gene sequences, and

optionally reporter sequences operably linked to expression control sequences. The adenovirus sequences forming the helper virus may be obtained from the sources identified above in the discussion of the pAd vector. Use of different Ad serotypes as helper viruses enables production of recombinant viruses containing the pAd $\Delta$  vector sequences in a capsid formed by the other serotype adenovirus. Use of these different Ad serotype helper viruses may also demonstrate advantages in recombinant virus production, stability and packaging.

The helper virus supplies the adenovirus sequences necessary for encapsidation of pAd $\Delta$ , including early genes E1, E2, E4, or fragments of a gene which perform the same or substantially the same function as the intact complete gene (i.e., functional fragments), and all remaining late, intermediate, structural and non-structural genes of the adenovirus genome, which are not present in the pAd $\Delta$  vector or provided by the cell line. Most suitably, the helper virus and cell line provide, at a minimum, adenovirus E1a, E1b, E2a, or functional fragments thereof. More preferably, the helper virus and cell line provide, E1a, E1b, E2a, E4, and VAI, or functional fragments of these genes (e.g., E4 ORF6) and the helper virus provides the adenoviral gene IX function. Preferably, the recombinant virus is an adenovirus and, most preferably, an adenovirus which replicates in the selected host cell. Alternatively, other suitable viruses, e.g., herpesviruses, may be used as helpers.

The selected helper virus is engineered to contain at least one, and preferably multiple, restriction enzyme sites (e.g., I-SceI). In one embodiment, the helper virus useful in the present invention contains three heterologous restriction enzyme sites. The presence of multiple restriction enzyme sites permits the helper virus to be digested by the rare-cutting restriction enzyme expressed in the cell, resulting in small fragments of helper virus which are readily distinguishable from and purified from the packaged recombinant virus (e.g., Ad $\Delta$ ) and any remaining unpackaged viral vector (e.g., pAd $\Delta$ ).

Most desirably, the helper virus contains a restriction enzyme site (e.g., a I-Sce-I site) located downstream of the packaging sequences, so that the helper virus is disabled for replication and packaging following digestion with the restriction enzyme expressed in the host cell. Other restriction enzyme sites may be readily

engineered into other suitable sites to disrupt functions which are no longer required following packaging of the viral vector and/or to permit monitoring of helper virus levels, e.g. by expression of a marker (i.e. reporter) sequence, which upon expression produces a detectable signal. Examples of suitable marker sequences are described  
5 above.

In one suitable embodiment, a rare-cutting restriction enzyme site (e.g., I-SceI) is placed upstream of the sequences encoding the marker, permitting monitoring of changing levels of intact helper virus by monitoring a decrease in the levels of detectable marker. In one desirable embodiment, the helper virus is composed of  
10 adenovirus 5' sequences, including a packaging signal, a first I-SceI site, a promoter, a second I-SceI site, a reporter gene, a third I-SceI site, and additional adenovirus sequences. Other suitable helper viruses may be readily designed and produced using the information provided herein.

#### 15 IV. Method of the Invention

In one embodiment, the present invention provides a method of producing helper-dependent viruses in a host cell which stably expresses the selected rare-cutting restriction enzyme (e.g., I-SceI). Suitably, the host cell is transfected with the recombinant viral vector, e.g., pAdΔ, and infected with the helper virus using  
20 conventional methods. See, generally, Sambrook et al, cited above. See also, the methods described in K.J. Fisher et al, *Virol.*, **217**:11-22 (1996). The host cell is then cultured under suitable conditions to permit encapsidation of recombinant viral vector in a first round of amplification. Suitably, where the rare-cutting restriction enzyme expression (e.g., I-SceI) is under the control of an inducible promoter, the inducing  
25 agent is added 6 to 72 hours, and preferably 24 hours, after infection of the cells with the helper virus.

In an alternative embodiment, the host cell is transiently transfected or infected with the sequences encoding the rare-cutting restriction enzyme (e.g., I-SceI). The host cell, provided with the recombinant viral vector and helper virus as  
30 described above, is then cultured in a similar manner to provide recombinant virus in a viral capsid.



Optionally, recombinant virus produced by this first round of viral amplification may then be recovered by conventional means. For example, the host cells may be harvested and lysed using known methods, e.g., three rounds of freeze (e.g., ethanol, dry ice) - thawing (e.g., 37°C). The cell lysate may then be centrifuged  
5 to remove cell debris. An advantage of the present invention is that, due to the difference in size between the recovered virus and the fragments into which the helper virus is cleaved by the restriction enzyme (e.g., I-SceI), the recovered virus is readily separated from the helper virus fragments. Where desired, the recovered virus may be subjected to further purification steps. See, Fisher et al, cited above.

10 Alternatively, the recovered lysate may be subjected to a second round of amplification, e.g., using the steps and host cells described above in the first round of amplification. This virus is useful for a variety of purposes known in the art.

The recombinant viruses produced according to the method of the invention are substantially free of helper virus contamination and are useful for a variety of  
15 purposes, which are well known to those of skill in the art.

The following examples are provided to illustrate methods for producing the compositions useful in the method of the invention and methods for performing the invention. Such examples do not limit the scope of the present invention. One skilled in the art will appreciate that although specific reagents and conditions are  
20 outlined in the following examples, modifications can be made which are meant to be encompassed by the spirit and scope of the invention.

#### Example 1 - Production of Helper Virus Containing I-SceI Sites

The helper adenovirus described in this example is termed Ad-3I since it carries three I-SceI sites in its genome.

##### 25 A. Production of pAd-linker-3I

The I-SceI coding region was synthesized by Anagen Inc. (CA, USA) and contains modified codons to optimize expression in mammalian cells. This

complete coding sequence for I-SceI is: SEQ ID NO:2: GGGGCGGCCG  
 CAGCCGCCAT ATGAAGAACA TCAAGAAGAA CCAGGTGATG  
 AACCTGGGCC CCAACAGCAA GCTGCTGAAG GAGTACAAGA  
 GCCAGCTGAT CGAGCTGAAC ATCGAGCAGT TCGAGGCCGG  
 5 CATCGGCCTG ATCCTGGGCG ACGCCTACAT CAGGAGCAGG  
 GACGAGGGCA AGACCTACTG CATGCAGTTC GAGTGGAAGA  
 ACAAGGCCTA CATGGACCAC GTGTGCCTGC TGTACGACCA  
 GTGGGTGCTG AGCCCCCCCC ACAAGAAGGA GAGGGTGAAC  
 CACCTGGGCA ACCTGGTGAT CACCTGGGGC GCCCAGACCT  
 10 TCAAGCACCA GGCCTTCAAC AAGCTGGCCA ACCTGTTTCAT  
 CGTGAACAAC AAGAAGACCA TCCCCAACAA CCTGGTGGAG  
 AACTACCTGA CCCCCATGAG CCTGGCCTAC TGGTTCATGG  
 ACGACGGCGG CAAGTGGGAC TACAACAAGA ACAGCACCAA  
 CAAGAGCATC GTGCTGAACA CCCAGAGCTT CACCTTCGAG  
 15 GAGGTGGAGT ACCTGGTGAA GGGCCTGAGG AACAAAGTTC  
 AGCTGAACTG CTACGTGAAG ATCAACAAGA ACAAGCCCAT  
 CATCTACATC GACAGCATGA GCTACCTGAT CTTCTACAAC  
 CTGATCAAGC CCTACCTGAT CCCCCAGATG ATGTACAAGC  
 TGCCCAACAC CATCAGCAGC GAGACCTTCC TGAAGTGATA TCGGG.

20 B. Production of Adenoviral Helper Virus

The pAd-linker-3I was constructed as follows. The synthetic I-SceI  
 oligonucleotide described in A above was inserted into pAd-linker1 at Bgl II and  
 Hind III [Fisher et al, cited above]. This plasmid contains an intact adenoviral  
 genome located on a pBR322 backbone. Another I-SceI site is cloned into pCI-AAT  
 25 ([Xiao et al, *J. Virol.*, 72:10222-10226 (1998)] following digestion with XhoI. The  
 Hind III-Cla I fragment of pCI-AAT with one I-SceI was then cloned into pAd-  
 linker1 with two I-SceI sites following digestion with EcoRV and ClaI, to obtain  
 pAd-linker-3I.

The helper virus Ad-3I was constructed by homologous recombination  
 30 between pAd-linker-3I and adenovirus DNA in 293 cells as described elsewhere [Gao

et al, *J. Virol.*, 70:8934-8943 (1996)]. The genome organization of Ad-3I is shown in Figure 2.

The resulting helper virus, Ad-3I, contains from 5' to 3': an adenovirus 5' ITR and packaging signal, a first I-SceI site, a CMV promoter, a second I-SceI site between the CMV promoter and the  $\alpha$ 1-antitrypsin cDNA, and a third I-SceI site between  $\alpha$ 1-antitrypsin and the other adenovirus early and late genes. The E1a and E1b genes are disrupted in this construct, which replicates in the presence of the E1 functions provided by the cell line.

The  $\alpha$ 1-antitrypsin gene driven by CMV is included in this helper virus to serve as a reporter gene. Digestion of the second I-SceI site (located between the CMV promoter and  $\alpha$ 1-antitrypsin cDNA) results in the loss of  $\alpha$ 1-antitrypsin gene expression. Additionally, digestion of any of the three I-SceI sites disables Ad-3I for replication and packaging since the 5' terminal repeat is thereby detached from helper adenovirus genome.

#### 15 C. Digestion of Ad-3I Helper Virus

The *in vitro* digestion of DNA by I-SceI was carried out using commercially available I-SceI (Boehringer Mannheim, Germany) under the conditions recommended by the vendor. To obtain Ad-3I DNA, the purified virus was digested with proteinase K and extracted from CsCl gradient purified adenovirus with phenol-chloroform before ethanol precipitation. The digested DNA was electrophoresed in 0.8% agarose gel. The size of the bands was identified. The complete digestion of Ad-3I results in four fragments of sizes of approximately 34 kb, 1.7 kb, 1.1 kb and 400 bp.

#### Example 2 - Production of Human Cell Line Expressing Functional I-SceI Enzyme

25 The synthesized I-SceI sequence described in Example 1A was fused at its N-terminus with a nuclear translocation signal (NLS) to ensure that the enzyme will reach the nucleus where adenovirus DNA replication takes place. This fusion was performed by inserting the I-SceI sequence downstream of a NLS in a suitable plasmid backbone. The NLS-I-SceI fragment was then removed by digestion with BspHI and NotI and the resulting fragment was cloned into plasmid, pEF-myc-nuc

30

(purchased from Invitrogen), to get pEF-SceI. pEF-myc-nuc contains a strong constitutive promoter derived from the human elongation factor 1 $\alpha$ . The 293-I-SceI cell line was constructed by transfecting plasmid pEF-SceI into 293 cells using lipofectAmine (Gibco BRL) selected under 200  $\mu$ g/ml of G418.

5        The G418 resistant clones (22) were selected and analysed for I-SceI expression following infection with Ad-3I at MOI 10. The Hirt DNA was extracted 36 hours post-infection. Southern analysis was performed using a human  $\alpha$ 1-antitrypsin cDNA probe. Fragments of 1.7 kb and 2.8 kb were obtained. The 1.7 kb fragment results from the digestion of I-SceI sites before and after  $\alpha$ 1-antitrypsin gene  
10      in Ad-3I. The results indicated that most G418 positive clones had the ability to digest helper Ad-3I genome in vivo.

These cell lines were maintained in Dulbecco's modified Eagle media (DMEM)-supplemented with 10% fetal calf serum in 5% CO<sub>2</sub> humidified environment at 37°C.

15        The infectious Ad-3I helper virus was used for screening the selected cell clones. The digestion of Ad-3I genome, which generates multiple small fragments in the Southern blot, is a indicator of the active I-SceI in the cell. Nearly all cell clones had the functional enzyme to digest the input adenovirus. On the other hand, in contrast to what observed in the in vitro digestion, the in vivo digestion seems not to  
20      be complete. The 2.8 kb fragment can be readily observed, which suggests that the second I-SceI site located between CMV promoter and  $\alpha$ 1-antitrypsin is not digested to 100%. This is probably due to the fact that cellular environment of mammalian cells is not the optimized condition as the *in vitro* digestion.

### Example 3 - Production of Recombinant Adenovirus

#### 25      A.      Generation of Helper-Dependent Vector

A helper-dependent vector, Ad- $\Delta$ -lacZ, was produced essentially as described in Fisher et al, *Virology*, 217:11-22 (1996). This vector contains the sequence from the left-end of Ad5 encompassing bp 1-360 (5' inverted terminal repeat (ITR), a lacZ minigene under the transcriptional control of the cytomegalovirus (CMV)  
30      immediate early enhancer/promoter, and sequence from the right end of Ad5

spanning bp 35,353 to the end of the genome (3' ITR). This plasmid was designed so that digestion with EcoRI releases the terminal ends of the Ad- $\Delta$ -lacZ genome from the plasmid backbone.

**B. Production of Recombinant Adenovirus**

5           The plasmid Ad- $\Delta$ -lacZ was linearized by EcoRI and transfected into 293 cells or a 293 I-SceI expression cell line of the invention. Helper virus Ad-3I was infected at MOI of 5. The cells were harvested at full cytopathic effect (CPE). After freezing and thawing the cells three times, the cell lysates were used for determination of virus titer by X-Gal staining, as described in Fisher et al, cited  
10 above. Cell lysates were also subjected to a second round of amplification to permit rescue of helper dependent vector Ad- $\Delta$ -lacZ, which was performed essentially as described by Fisher et al, cited above.

**C. I-SceI Cell Lines Enhanced Helper Dependent Adenovirus Production**

          The performance of the 293 I-SceI cell line in production of a  
15 recombinant adenovirus deleted of all adenoviral genes is presented in Fig. 4. The deleted Ad vector, EcoRI-digested pAd- $\Delta$ -lacZ (10  $\mu$ g), was transfected in  $1 \times 10^5$  cells of each of three cell lines: a conventional 293 cell line and two independent clones of 293-I-SceI (termed (a) and (b)). After transfection, the cells were infected with Ad-3I helper virus at MOI 5. The cell lysates were harvested 96 hours after  
20 infection. A portion of the cell lysates were used to determine the titer of Ad- $\Delta$ -lacZ virus titer for the first round of amplification. The remainder of the cell lysate was used for a second round of amplification in the same cell lines. The titer is shown as lacZ forming unit (LFU) per field under microscopy under identical conditions. See Fig. 4.

25           Interestingly, the initial rescue of helper dependent viruses was better in 293 cells while subsequent amplification of helper virus is better in 293-I-SceI cell lines. This may reflect the importance in the initial rescue step of adequate amounts of helper virus. In the 293-I-SceI cell line, the amount of helper virus is decreased because of I-SceI digestion. In the amplification procedure, the balance of helper  
30 virus and vector is more critical.

A further characterization of the Ad-3I-293-I-SceI system is shown in Fig. 5. In this experiment, the same amounts of helper virus and EcoRI-digested plasmid Ad- $\Delta$ -lacZ were used to infect the same amounts of 293 cells, 293-I-SceI(a) cells and 293-I-SceI(b) cells (two different clones). The amount of helper virus was  
5 decreased in the 293-I-SceI cell line. However, the helper virus yield is increased 10-100 fold in a simple step of amplification.

#### Example 4 - Production of Helper-Dependent Recombinant Adenovirus

##### A. Production of rAAV vector expressing I-SceI enzyme

The plasmid pAAV-SceI was constructed by cloning the blunt ended  
10 EF-nuc-I-SceI cassette from pEF-SceI into pSub201 at the XbaI site. Infectious AAV-SceI was generated using the techniques described previously [Xiao, *J. Virol.*, 72:10222-10226 (1998)].

##### B. Production of Helper-Dependent Recombinant Adenovirus

293 cells ( $1 \times 10^5$ ) are transfected with 10  $\mu$ g of EcoRI-digested pAd-  
15  $\Delta$ -lacZ and rAAV-SceI (MOI 10). After transfection, the cells are infected with Ad-3I at MOI 5. The cell lysates are harvested 96 hours after infection.

#### Example 5 - Characterization of 293-I-SceI Cell Line and rAAV-SceI Vectors

To further study the helper-dependent rAd virus production methods of the invention, the levels of reporter gene expression ( $\alpha$ 1-antitrypsin gene expression)  
20 after Ad-3I infection (MOI from 0.1, 1, 10, 100, 200) in media from either 293 cells, the 293-I-SceI cell line, or 293 cells with rAAV-EF-I-SceI (MOI 10) co-infection were monitored at various time points by ELISA which was performed as described by [Xiao et al, *J. Virol.*, 72:10222-10226 (1998)].

A Southern blot of the Hirt DNA was performed using a probe specific for  
25 human  $\alpha$ 1-antitrypsin cDNA. DNA fragments of 1.7 kb, 2.8 kb and 32 kb were obtained. These fragments are indicative of digestion with the I-SceI. The Hirt DNA was extracted 24 hours post infection and electrophoresed in 0.8% agarose gel. The results clearly showed that replication of helper virus, Ad-3I, was lowered dramatically in the presence of I-SceI gene expression in the cell line.

Ad-3I genome is disabled by I-SceI restriction digestion, which most likely causes the decrease in the copy number of the  $\alpha$ 1-antitrypsin gene available for transcription. The Southern blot also further confirmed that this is the case. In either 293-I-SceI cell or with rAAV-I-SceI co-infection, the replication of Ad-3I is also  
5 reduced. This is evident since the large fragment (>30kb) in the presence of I-SceI expression is less than that generated in 293 cells. The digested small fragments are also evident in the blots. Moreover, this experiment suggested the I-SceI enzyme delivered by rAAV vector was more potent than the I-SceI enzyme expressed from 293-I-SceI cell line. This is probably due to the fact that infectious rAAV transduced  
10 the 293 cells with more copies of I-SceI gene than are present in the integrated counterpart (i.e., the 293-I-SceI cell line).

All publications cited in this specification are incorporated herein by reference herein. While the invention has been described with reference to a particularly preferred embodiment, it will be appreciated that modifications can be made without  
15 departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. A method for producing a virus from a vector which requires helper virus for viral packaging, said method comprising the steps of:
  - (a) providing a host cell expressing a rare-cutting restriction enzyme heterologous to the host cell;
  - (b) introducing into the host cell with a recombinant viral vector comprising a minigene containing a transgene encoding a selected protein and regulatory sequences useful for directing expression of said protein;
  - (c) introducing into the host cell with a recombinant helper virus engineered to contain multiple restriction sites for the rare-cutting restriction enzyme, wherein the restriction enzyme is heterologous to the helper virus; and
  - (d) culturing the host cell transfected with the recombinant viral vector and helper virus under conditions which permit packaging of the recombinant viral vector into a virus,  
wherein the helper virus and the host cell provide to the recombinant virus vector the necessary viral genes for viral packaging.
2. The method according to claim 1, wherein the cell line further expresses helper functions.
3. The method according to claim 1, wherein the restriction enzyme is I-SceI.
4. The method according to claim 1, wherein the cell line expresses the restriction enzyme in the nucleus.
5. The method according to claim 1, wherein the cell line expresses the restriction enzyme under the control of an inducible promoter.



6. The method according to claim 1, wherein the cell line expresses the restriction enzyme under the control of a constitutive promoter.
7. The method according to claim 1, wherein the viral vector is selected from the group consisting of an adenoviral vector, an adeno-associated virus vector, a retroviral vector, and a lentiviral vector.
8. The method according to claim 7, wherein the viral vector is a recombinant adenovirus vector comprising adenovirus 5' cis-elements and 3' cis-elements necessary for packaging and encapsidation flanking the minigene, in the absence of other functional adenovirus genes.
9. The method according to claim 7, wherein the viral vector is a recombinant adenovirus vector comprising adenovirus 5' and 3' cis-elements necessary for packaging and encapsidation in a head-to-tail orientation.
10. The method according to claim 9, wherein the adenovirus 5' cis-elements comprise the 5' inverted terminal repeats, or a functional fragment thereof.
11. The method according to claim 9, wherein the adenovirus 3' cis-elements comprise the 3' inverted terminal repeats, or a functional fragment thereof.
12. The method according to claim 1, wherein the recombinant helper virus is selected from the group consisting of a recombinant adeno-associated virus, a retrovirus, a lentivirus, and a recombinant adenovirus.
13. The method according to claim 1, wherein the recombinant helper virus comprises three I-SceI restriction enzyme sites.

14. The method according to claim 13, wherein the recombinant helper virus is disabled for replication and packaging following digestion with the I-SceI enzyme expressed in the host cell.
15. A recombinant virus produced by the method of claim 1.
16. A stable mammalian cell line which expresses a rare-cutting restriction enzyme in the cell nucleus.
17. The cell line according to claim 16, wherein the restriction enzyme is I-SceI.
18. The cell line according to claim 16, wherein the cell line is a human cell line.
19. A recombinant helper virus useful in helper-dependent production of a recombinant viral vector, wherein said helper virus is engineered to contain a rare-cutting, heterologous, restriction enzyme site downstream of the packaging signal.
20. The recombinant helper virus according to claim 19, wherein the restriction enzyme is I-SceI.
21. The recombinant helper virus according to claim 20, wherein said helper virus comprises multiple I-SceI sites.
22. The recombinant helper virus according to claim 19, wherein said helper virus is a recombinant adenovirus.
23. The recombinant helper virus according to claim 22, wherein said helper virus comprises: a packaging signal, a first I-SceI site, a promoter, a second I-SceI site, a reporter gene, a third I-SceI site, and adenoviral sequences.

24. A method for the production of virus from a viral vector which requires a helper virus for packaging, said method comprising the steps of:

- (a) introducing into a host cell with a recombinant viral vector comprising a minigene containing a transgene encoding a selected protein and regulatory sequences useful for directing expression of said protein;
- (b) introducing into the host cell with a recombinant helper virus engineered to contain a rare-cutting restriction site for a restriction enzyme heterologous to the helper virus and host cell;
- (c) delivering the restriction enzyme to the host cell; and
- (d) culturing the host cell transfected with the recombinant viral vector and helper virus under conditions which permit packaging of the recombinant adenovirus vector in a viral particle;

wherein the helper virus and the host cell provide to the recombinant viral vector the necessary viral genes for viral packaging; and

wherein the restriction enzyme cleaves the recombinant helper virus following generation of the recombinant adenovirus vector.

25. The method according to claim 24, wherein the restriction enzyme is delivered to the host cell via a recombinant virus encoding the restriction enzyme under the control of regulatory sequences which direct expression of the restriction enzyme in the host cell.

26. The method according to claim 25, wherein the recombinant virus encoding the restriction enzyme is a recombinant adeno-associated virus, which is delivered at a multiplicity of infection of about 100 rAAV genome particles.

27. A cell lysate comprising a recombinant virus, wherein said recombinant virus is substantially free of intact helper virus.

28. A recombinant virus purified from the cell lysate of claim 27.

1 / 3

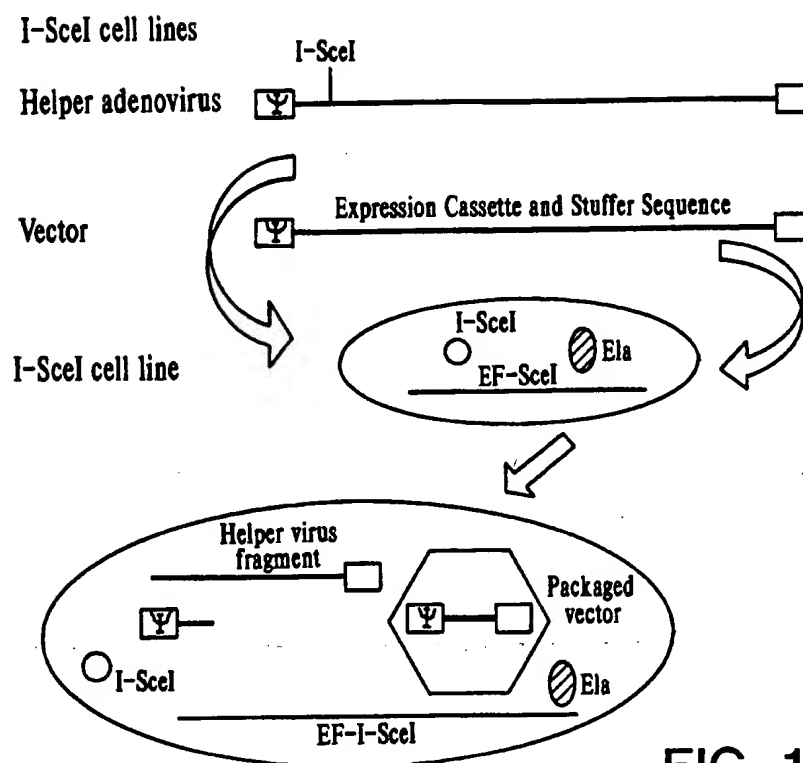


FIG. 1A

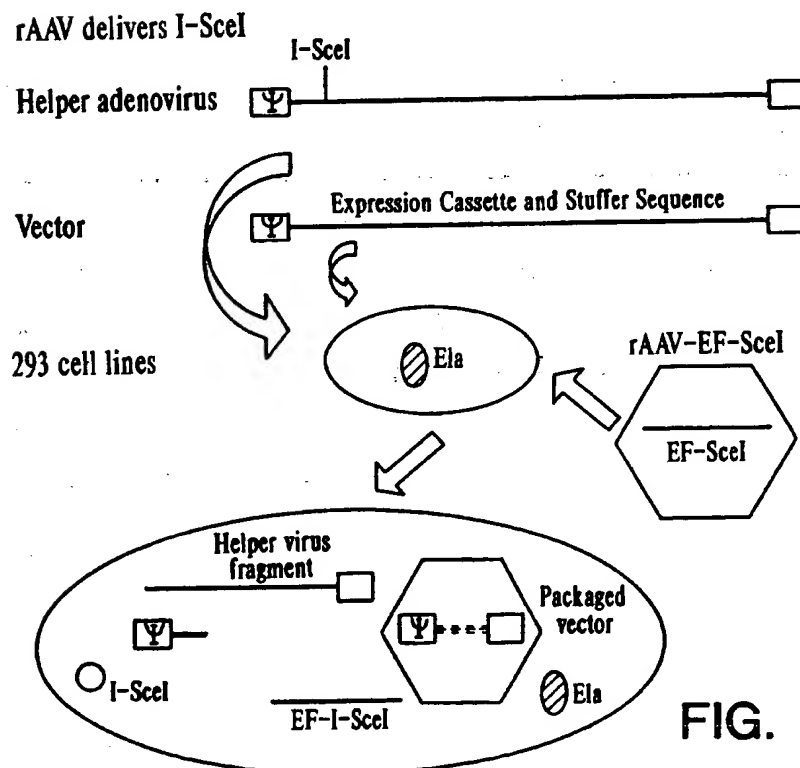


FIG. 1B

2 / 3

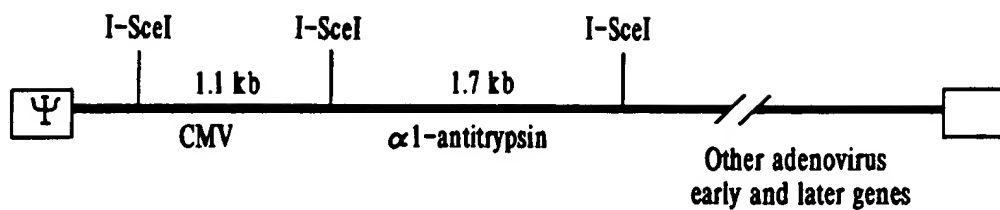


FIG. 2

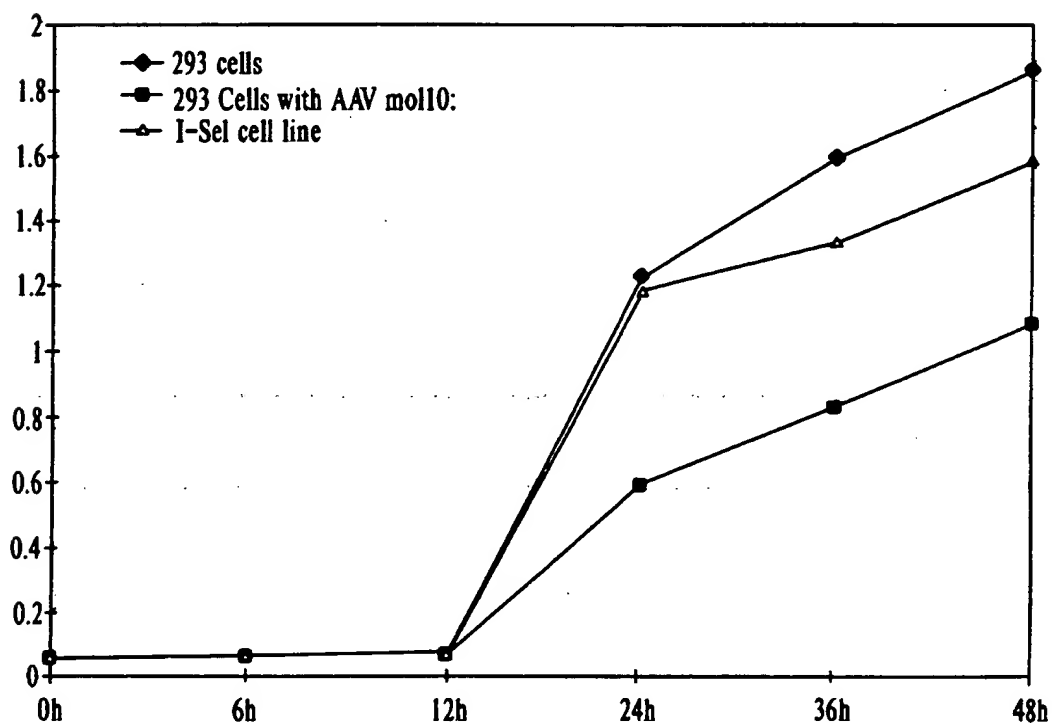


FIG. 3

3 / 3

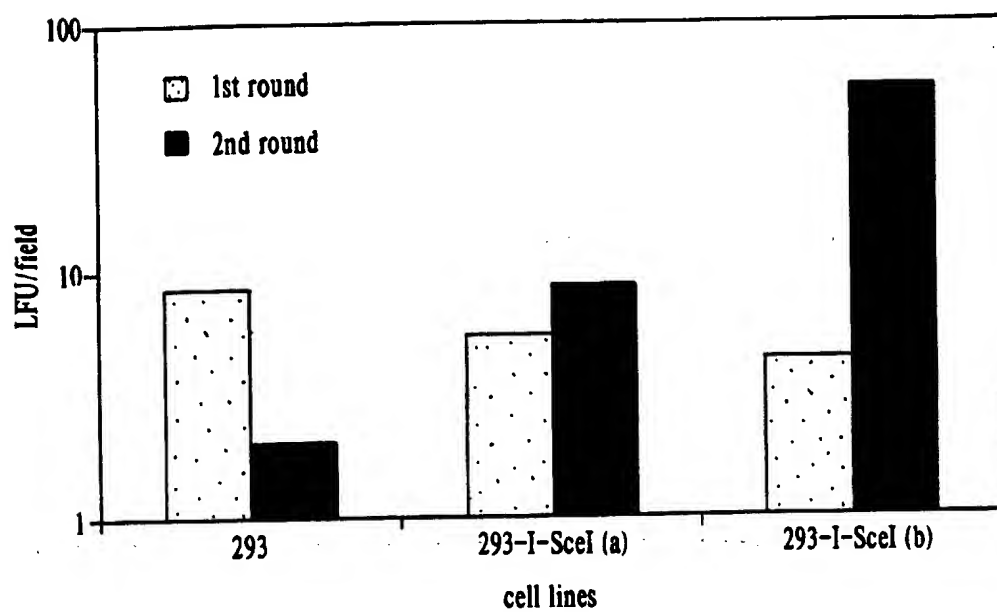


FIG. 4

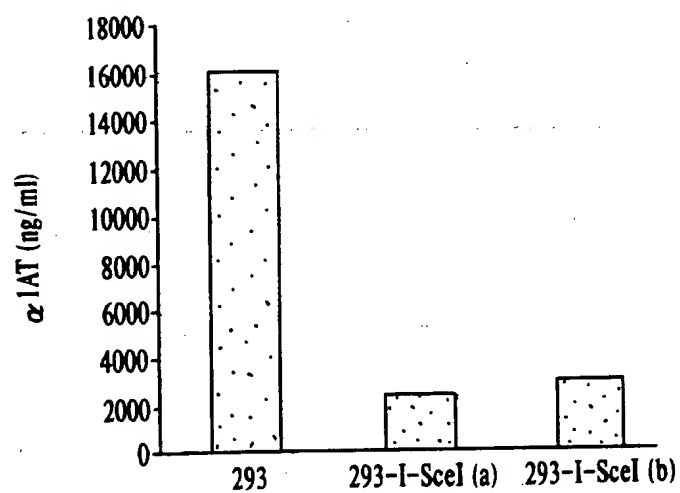


FIG. 5

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/00415

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/86 C12N5/10 C12N9/22 C12N7/01

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| X          | WO 96 14408 A (PASTEUR INSTITUT ; UNIV PARIS CURIE (FR)) 17 May 1996 (1996-05-17)<br>page 28, paragraph 2<br>examples 4,5<br>page 62, line 1 - line 2<br>page 62, paragraph 3<br>page 64, line 7<br>page 64, paragraph 4 | 16-18                 |
| X          | WO 94 12649 A (GENZYME CORP)<br>9 June 1994 (1994-06-09)<br>example 11   | 27, 28                |
| A          | EP 0 711 829 A (VIAGENE INC)<br>15 May 1996 (1996-05-15)<br>page 21, line 18 - line 27   | 1-28                  |

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*A\* document member of the same patent family

Date of the actual completion of the international search

31 May 2000

Date of mailing of the international search report

07/06/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3018

Authorized officer

Mandl, B

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/00415

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|---------------------|----------------------------|---------------------|
| WO 9614408 A                              | 17-05-1996          | US 5792632 A               | 11-08-1998          |
|   |                     | CA 2203569 A               | 17-05-1996          |
|   |                     | EP 0791058 A               | 27-08-1997          |
|   |                     | JP 10508478 T              | 25-08-1998          |
|   |                     | US 5948678 A               | 07-09-1999          |
|   |                     | US 5866361 A               | 02-02-1999          |
| WO 9412649 A                              | 09-06-1994          | US 5670488 A               | 23-09-1997          |
|   |                     | AU 4365597 A               | 12-02-1998          |
|   |                     | AU 680459 B                | 31-07-1997          |
|   |                     | AU 5734994 A               | 22-06-1994          |
|   |                     | CA 2145641 A               | 09-06-1994          |
|   |                     | EP 0673431 A               | 27-09-1995          |
|   |                     | EP 0905253 A               | 31-03-1999          |
|   |                     | EP 0911413 A               | 28-04-1999          |
|   |                     | JP 8503855 T               | 30-04-1996          |
|   |                     | US 5882877 A               | 16-03-1999          |
| EP 0711829 A                              | 15-05-1996          | EP 0716148 A               | 12-06-1996          |
|   |                     | EP 0814154 A               | 29-12-1997          |
|   |                     | EP 0982405 A               | 01-03-2000          |
|   |                     | AU 3682699 A               | 07-10-1999          |
|   |                     | AU 703653 B                | 01-04-1999          |
|   |                     | AU 5645098 A               | 04-06-1998          |
|   |                     | AU 690583 B                | 30-04-1998          |
|   |                     | AU 7835894 A               | 03-04-1995          |
|   |                     | CA 2158937 A               | 23-03-1995          |
|   |                     | EP 0694070 A               | 31-01-1996          |
|   |                     | FI 954601 A                | 23-02-1996          |
|   |                     | JP 9503657 T               | 15-04-1997          |
|   |                     | NO 953901 A                | 04-01-1996          |
|   |                     | WO 9507994 A               | 23-03-1995          |
|   |                     | US 6015686 A               | 18-01-2000          |
|   |                     | US 5814482 A               | 29-09-1998          |
|   |                     | US 5843723 A               | 01-12-1998          |
|   |                     | US 5789245 A               | 04-08-1998          |
|   |                     | US 6015694 A               | 18-01-2000          |



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**